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TRANSLATION NO. 2429

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JUN 24 1969

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T-695-1

2421

IMMUNOCHEMICAL INVESTIGATIONS IN PLAGUE

--(Species affiliation of the Iolatan strains)

Following is the translation of an article by V. I. Veynblat, Turkmen Antiplague Station (Head, A. G. Serasimenko) and the All-Union Antiplague Institute "Mikrob" (Director - Doctor of Medical Sciences N. I. Nikolayev), published in the Russian-language periodical Zdrav. Turk. (Public Health in Turkmenia), 2: 37--41, 1968.

Over a period of a number of years in the Tedzhen-Murgabskiy interfluvial area in Turkmenistan a considerable number of plague-like cultures of bacteria have been isolated from sick gerbils. In particular this concerns the Iolatan or Badyzsk strains (A. K. Akiyev et al., 1960). Up till now the species affiliation of the latter has not been clear, although their precise classification has great significance for the epizootological and epidemiological characteristics of the territory of southeastern Turkmenia.

The purpose of the present investigation is a study of the antigenic relationship of microbes of the Iolatan strains with the bacteria of plague and pseudotuberculosis of rodents.

Materials and methods. Bacteria of the Iolatan strains were subjected to immunochemical analysis for the presense of specific antigens of the plague microbe and serologically active polysaccharide (hapten). In addition to this their fibrinolytic activity was studied. The investigation was carried out on Iolatan strains 746, 48, 1953, and 1962, isolated from wild rodents in 1961 and 1963, and smooth variants 746 and 1953, obtained in the laboratory by Ye. M. Punskiy. As a control we used the plague strains EV N12G and 533 (virulent, continental variant) and the typical strains of pseudotuberculosis of rodents - 34S and 1-R. The microbes were incubated on Hottinger agar at 37° for 3 days, then washed with physiological solution, killed, and dried with cooled acetone. For exposure of fibrinolytic activity we used 1--10 billion suspensions 10^8 /ml of live bacteria, incubated at 28° on Hottinger agar for 3 days. In the determination of Fraction I of Baker et al. we used the reactions of antibody neutralization (Levi, Momot, 1961) and diffusion precipitation in agar (V. L. Pustovalov et al., 1962). The presence of Fraction I in the microbes was judged also by their ability to cause the formation of antibodies to it in the organism of rabbits. The sera of hyperimmunized animals were investigated in the reactions of passive hemagglutination (RPH) and inhibition of passive hemagglutination (Yu. G. Suchkov, 1963). For setting

up these reactions we used formalinized sheep erythrocytes which were treated with tannin and Fraction 1.

* [Trans. note. This unit has not been definitely established. Tel evidently refers to tello or body.]

The presence of serologically active polysaccharide in the microbes was established with the help of the reaction of polysaccharide hemagglutination (Ye. M. Bakhrakh et al., 1964).

Group antigens of bacteria were identified in the reaction of diffusion precipitation in agar. We also used the agglutinating plague serum of the "Mikrob" Institute and sera against the bacteria of Flexner dysentery, typhoid, paratyphoid A, and salmonella Norther and Morgan.

The high toxicity of Fraction 2 for white mice made it possible to use the biological method for its titration. Separate groups of white mice were given intraperitoneally suspensions of acetone-dried bacteria or aqueous-saline extracts of them. The latter were prepared by the method of Baker et al. (1952).

Determination of the fibrinolytic activity of the bacteria was carried out in the reactions of fibrinogenolysis and fibrinolysis. (G. Ya. Yaromyuk, 1964). Ingredients of the reactions were fibrinolytic fractions of the plague microbe, highly purified fibrinogen, and thrombin, which were kindly given to us by I. V. Domoradskiy.

Microbes of the Iolatan strains and pseudotuberculosis of rodents gave negative results in the antibody neutralization reaction. Analogous findings were obtained with aqueous-saline extracts of bacteria. However, in the latter case an exception was an extract from microbes of strain 746, which reacted positively in the antibody neutralization reaction (to a dilution of 1:500). From this extract several fractions were isolated by subsequent saturation with ammonium sulfate. Solutions of 3 fractions, precipitated with 30, 40, and 67% concentration of ammonium sulfate in the extract, were investigated in the antibody neutralization reaction (RNA). Here their titers comprised accordingly 1:240, 1:160, and 1:120. Each fraction separately was subjected to purification by means of repeated precipitation with ammonium sulfate. After each reprecipitation the titer of the fraction was checked in the RNA. Instead of the expected increase in titer, as this took place during an analogous fractionation of water-soluble antigens of the plague microbe (a positive result in the RNA of plague antigens was preserved up to a dilution of them of 1:10⁶--1:10⁷), the titer of the investigated fractions was reduced rapidly and after 3 reprecipitations it became equal to 0, and the resulting fractions disappeared completely in the process of reprecipitation.

In the reaction of diffusion precipitation in agar the same extract did not produce a precipitation line which would coincide with the corresponding Fraction I.

Investigations in the reaction of passive hemagglutination of sera obtained from hyperimmunized rabbits showed that microbes of the Iolatan strains do not stimulate the formation of antibodies to Fraction I in the organism of these animals. Though the sera of rabbits, immunized with microbes of strains 746-R and 1-R, yielded a positive result in the RPH in a dilution of 1:40--1:240, these results turned out to be nonspecific, which was established with the help of a reliable control of specificity - the reaction of inhibition of passive hemagglutination.

In the reaction of polysaccharide hemagglutination microbes of the Iolatan strains, pseudotuberculosis of rodents, and plague reacted positively (see Table 1).

Table 1

Reaction of polysaccharide hemagglutination with microbes of the Iolatan strains, pseudotuberculosis of rodents, and plague

<div> <div>(a)</div> <div>Принадлежность бактерий</div> </div>	<div> <div>(b)</div> <div>Чувствительность реакции полисахаридной гемагглютинации с гаптеном, приготовленным из бактерий (мг)</div> </div>				
	0,5	0,25	0,12	0,06	0,03
(c) Иолатанские	+4	+2	—	—	—
(d) псевдотуберкулез	+4	+4	+4	+2	—
(e) Чумные	+4	+4	+4	+2	—

Key: (a) Affiliation of bacteria; (b) Sensitivity of the reaction of polysaccharide hemagglutination with hapten, prepared from bacteria (mg); (c) Iolatan; (d) Pseudotuberculosis; (e) Plague.

Microbes of plague and pseudotuberculosis of rodents preserved serologically active polysaccharide (hapten) in approximately equal quantities, and Iolatan strains - somewhat less. No differences were observed between the content of serologically active polysaccharide in rough and smooth variants of microbes of the Iolatan strains.

Results of the investigation of microbes for the presence of fraction (2 toxic) are presented in Table 2.

As can be seen from data in Table 2, acetone-treated bacteria

of Iolatan strains in large doses can cause the death of a portion of the white mice. During an autopsy of them inflammation of the vessels of the subcutaneous cellular tissue, hyperemia of the lungs, and enlargement of the spleen were revealed. Hyperemia of the lungs was noted more often in white mice which died from microbes of the Iolatan strains than from plague bacteria.

Table 2

Toxic effect of bacteria of the Iolatan strains of pseudotuberculosis of rodents and plague on white mice

Количество бактериаль- ной массы в мг сухого веса	746		1953		1962	43	1	34	EB	533
	R	S	R	S	R	R	R	S	R	R
8	4/2	4/3	4/2	4/2	4/1	4/2	4/0	4/0	4/4	4/4
4	4/1	4/1	4/1	4/1	4/0	4/0	4/0	4/0	4/4	4/4
2	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/4	4/4
1	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/4	4/4

Note. Numerator - total number of animals; denominator - number of animals which died.

Key: (a) Amount of bacterial mass in mg of dry weight.

The death of mice from the intraperitoneal $\sqrt{7}$ here there are three letters which have no meaning administration of suspensions of microbes of the Iolatan strains could not be caused by the water-soluble Fraction 2, but by some other toxic components of these bacteria. In order to clear up the reason for the death of the animals we prepared aqueous-saline extracts of microbes of Iolatan strains and plague. The white mice were given amounts of extract which corresponded to 20 mg of dry bacteria of the Iolatan strains or 1 mg of plague microbes. Death of the animals set in only under the influence of the extract from plague bacteria. Thus the toxic component of bacteria of the Iolatan strains was concentrated in the water-insoluble segment of the microbial cell, which pointed to the non-identity of the toxin of bacteria of Iolatan strains to the toxin of plague bacteria (Fraction 2).

Investigation of the microbes with the help of the reaction of fibrinolysis and fibrinogenolysis showed that in contrast to plague bacteria the microbes of Iolatan strains and pseudotuberculosis of rodents did not possess a fibrinolytic activity.

Results of the investigations, conducted for exposing group antigens with the help of the reaction of diffusion precipitation in agar, are depicted in Table 3.

Table 3

Reactions of diffusion precipitation in agar with bacteria of plague, pseudotuberculosis of rodents, and Iolatan strains

Вид агглютинирующих сывороток	Число преципитационных линий, образованных антителами сывороток и водорастворимыми антигенами микробов штаммов									
	1953		746		43	1962	1-Р	3-1-5	ЕВ	53
	R	S	R	S						
Чумная	4	5	4	4	3	5	5	5	7	7
Кишечно-тифозная группа бактерий	—	—	—	—	—	—	—	—	—	—

Key: (a) Type of agglutinating sera; (b) Number of precipitation lines formed by antibodies of sera and by water-soluble antigens of microbes of strains: (c) Plague; (d) Enteric-typhoid group of bacteria.

In the reaction of diffusion precipitation in gel with anti-plague agglutinating serum the microbes of the Iolatan strains and pseudotuberculosis of rodents formed up to 4--5 precipitation lines, coinciding with the corresponding lines of precipitation of plague antigens and antibodies of agglutinating serum. Not one of these lines fused with the zone of precipitation belonging to Fraction I. Apparently the stated precipitation lines were formed by antigens which are common for bacteria of pseudotuberculosis of rodents, Iolatan strains, and plague.

We conducted still one more test, in which a solution of antigen and serum was placed in the agar holes for the reaction of diffusion precipitation not once, but in lots over a period of several days (V. L. Rustovalov et al., 1962) and in greater amounts than in the first experiment. Here additional zones of precipitation appeared and the total number of precipitation lines increased in the holes with plague microbes up to 15, with pseudotuberculosis bacteria - up to 12, and with microbes of the Iolatan strains - up to 10. However, the number of coinciding precipitation lines, formed by related antigens of bacteria of various types and antibodies of serum, remained the same as during the single application of antigens and serum in the agar holes.

Parallel with the study of antigens of the Iolatan strains which were common with antigens of plague and pseudotuberculosis microbes we conducted an experiment for investigation of the antigenic community of Iolatan, plague, and pseudotuberculosis bacteria

with microbes of the enteric-typhoid group. For this purpose the reaction of diffusion precipitation in agar was set up with microbes of all the strains used in the investigation and with commercial agglutinating sera: Flexner dysentery, typhoid, paratyphoid A, and salmonella Hertner and Morgan. The results of the experiment turned out to be negative, since not in one case did we observe a precipitation reaction between antigens of bacteria and antibodies of serum.

It is necessary to note that for obtaining more reliable results all the tests with the reaction of diffusion precipitation in agar were conducted at the same time, with one series of agar, and in the same routine and on the same number of Petri dishes. This permitted the multiple doubling of each individual result.

Conclusions

Microbes of the Iolatan strains have antigens which are common with bacteria of plague and pseudotuberculosis of rodents. At the same time the antigen composition of bacteria of Iolatan strains differed considerably from the antigen composition of microbes from the enteric-typhoid group. Bacteria of the Iolatan strains did not contain the antigen--fraction I which is specific for the plague microbe. It is true that microbes of strain 746 caused the formation, in the organism of rabbits, of antibodies which, in the RPH, agglutinated sheep erythrocytes which were sensitized with Fraction I. However, the negative results obtained in the investigation of the sera from these rabbits in the reaction of inhibition of passive hemagglutination indicate the insufficient specificity of antibodies which agglutinated diagnostic agent. Microbes of strain 746 also contain a protein factor, large amounts of which may bind antibodies to Fraction I in the antibody neutralization reaction. It is possible that to some degree this component possesses an antigen relationship with Fraction I, but not sufficient enough to cause the formation of antibodies which are completely identical to antibodies to Fraction I in the organism of rabbits. Apparently the antigen from bacteria of the Iolatan strain 746 which we investigated is analogous to the antigen discovered by Kvan and associates in 1965 in certain strains of pseudotuberculosis of rodents.

In microbes of the Iolatan strains there is an absence of fibrinolytic activity, which is one of the rather constant features of the plague microbe and never occurs in pseudotuberculosis microbes (G. A. Yaromyuk, 1963).

Microbes of the Iolatan strains do not contain the antigen which determines the toxicity of Fraction 2 (Baker et al., 1952) for white mice and rats. It is known that the toxin of the plague microbe is specific and differs from the toxin of bacteria of pseudotuberculosis of rodents, (Knapp, 1959). However, microbes of

of lolatan strains, just as certain types of pseudotuberculosis bacteria, contain a substance which is toxic for white mice. It is localized in the water-insoluble portion of the bacterial cell.

Our own observations and an analysis of data from the literature on the lolatan strains (A. K. Akiev et al., 1961; A. V. Rusanov, 1963; B. Ye. Pinskiy et al., 1964; G. M. Golkovskiy, 1965), plague bacteria (V. M. Tumanskiy, 1958; I. V. Domoradskiy et al., 1960; Burrows, 1963; Chen, 1965; and others), and microbes of pseudotuberculosis of rodents (Knapp, 1959; A. M. Antonov, 1960; Kvan et al., 1965) showed that in the investigated groups of bacteria there is much in common. Particularly apparent is the identity of a large share of most important features (biochemical, immunocchemical, biological, etc.) of the lolatan and pseudotuberculosis bacteria. Apparently the microbes of the lolatan strains are a variant of *P. pseudotuberculosis* rod prieti, which during the course of evolutionary adaptation to environment have lost certain typical properties. This led to difficulties in the identification of bacteria of the lolatan strains and the carrying out of differential diagnosis of them with bacteria of plague and pseudotuberculosis of rodents.